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Delineation of DNA replication time zones by fluorescence *in situ* hybridization

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Fluorescence *in situ* hybridization has been used to visualize specific genomic DNA sequences in interphase nuclei. In normal diploid cells, unreplicated DNA segments give singlet hybridization signals while replicated loci are characterized by doublets. The distribution of these two patterns in unsynchronized cell populations can be used to determine the S phase replication time of any DNA sequence. The validity of this approach was established by analyzing genes whose replication profiles in expressing and non-expressing cells had been determined previously by conventional methods. Using this technique it has been possible to map the replication timing topography of the DNA within and flanking the cystic fibrosis (CF) gene locus on chromosome 7. The gene itself is located within a defined time zone which is ~500 kb in length and is under developmental control. It is early replicating in cells which express CF but late replicating in other cell types. These time zones probably represent basic units of chromosome structure.

Key words: chromosome structure/interphase nuclei/replication bands

Introduction

Replication of DNA in the animal cell genome takes place in a temporally ordered fashion during S phase. Cells labeled with BrdU for various periods of time exhibit a pattern of incorporation on metaphase chromosomes that defines a series of replication bands which appear in a reproducible programmed manner over the length of the genome (Hand, 1978). Although the exact boundaries of any of these bands have not been identified, they generally contain several megabases of DNA and it has been estimated that each is made up of 10–20 individual replicons which must be activated in a coordinated manner (Hand, 1978; Holmquist, 1987). This latter idea is supported by fiber autoradiography studies which show large clusters of replicons active at any given time in the cell cycle (Edenberg and Huberman, 1975).

The replication timing of individual genes in different cell lines has also been examined extensively. This is usually carried out by Southern blot hybridization to newly replicated BrdU-labeled DNA which has been isolated from cells in different stages of the S phase (Braunstein *et al.*, 1982). Every gene sequence so far studied has a defined time of replication within the cell cycle. Almost all housekeeping genes replicate within the first half of S in many cell types and the same is true for some tissue specific genes. Other tissue specific gene sequences have a developmentally regulated pattern of replication whereby they undergo DNA synthesis early in expressing cells, but late in non-expressing cell types (Goldman *et al.*, 1984; Hatton *et al.*, 1988; reviewed by Holmquist, 1987). The most extensively studied gene region of this type is the human β-globin domain, where over 200 kb of DNA is early replicating in K-562 erythroleukemia cells, but late replicating in cultured lymphocytes and in HeLa cells (Epner *et al.*, 1988; Dhar *et al.*, 1988). Although the boundaries of this timing zone have not been defined, fusion experiments between mouse erythroleukemia cells and normal or mutant human lymphocytes suggest that the far upstream locus activation region (LAR) may be involved in regulating both the chromatin structure and replication timing of this region (Forrester *et al.*, 1990).

In this manuscript we describe a new approach to study replication timing that does not require the prelabeling or synchronization of cells and is based on the ability to visualize specific replicated gene segments in interphase nuclei by *in situ* hybridization. This method has been used to define the replication timing boundaries in the human cystic fibrosis (CF) gene region on chromosome 7. Our results indicate that this gene is located within a single distinct replication time zone which is under developmental control and thus switches from late to early in cells which actively express the CF protein.

Results

Measurement of replication timing by in situ hybridization

Although *in situ* hybridization has been used extensively to map the location of specific gene sequences on metaphase chromosomes, it is only recently that technical refinements have made it possible to delineate individual genes in interphase nuclei (Lawrence *et al.*, 1988; Trask and Hamlin, 1989). By combining non-isotopically labeled probes with fluorescence detection methods, genetic loci can be visualized with high spatial resolution. Furthermore, digital imaging microscopy and sensitive photon counting cameras facilitate quantitative analysis and the application of image processing techniques to enhance image quality. One can also increase the strength of the hybridization signal by using high concentrations of probe DNA (Lawrence *et al.*, 1988) and by using large phage or cosmid constructs whose repetitive sequence components are eliminated by prehybridization with Cot-1 DNA or homologous total genomic DNA (Landegent *et al.*, 1987; Lichter *et al.*, 1988). These critical observations suggested that *in situ* hybridization may provide a new tool for measuring the replication timing of specific genomic DNA segments. Indeed, when a random population of nuclei

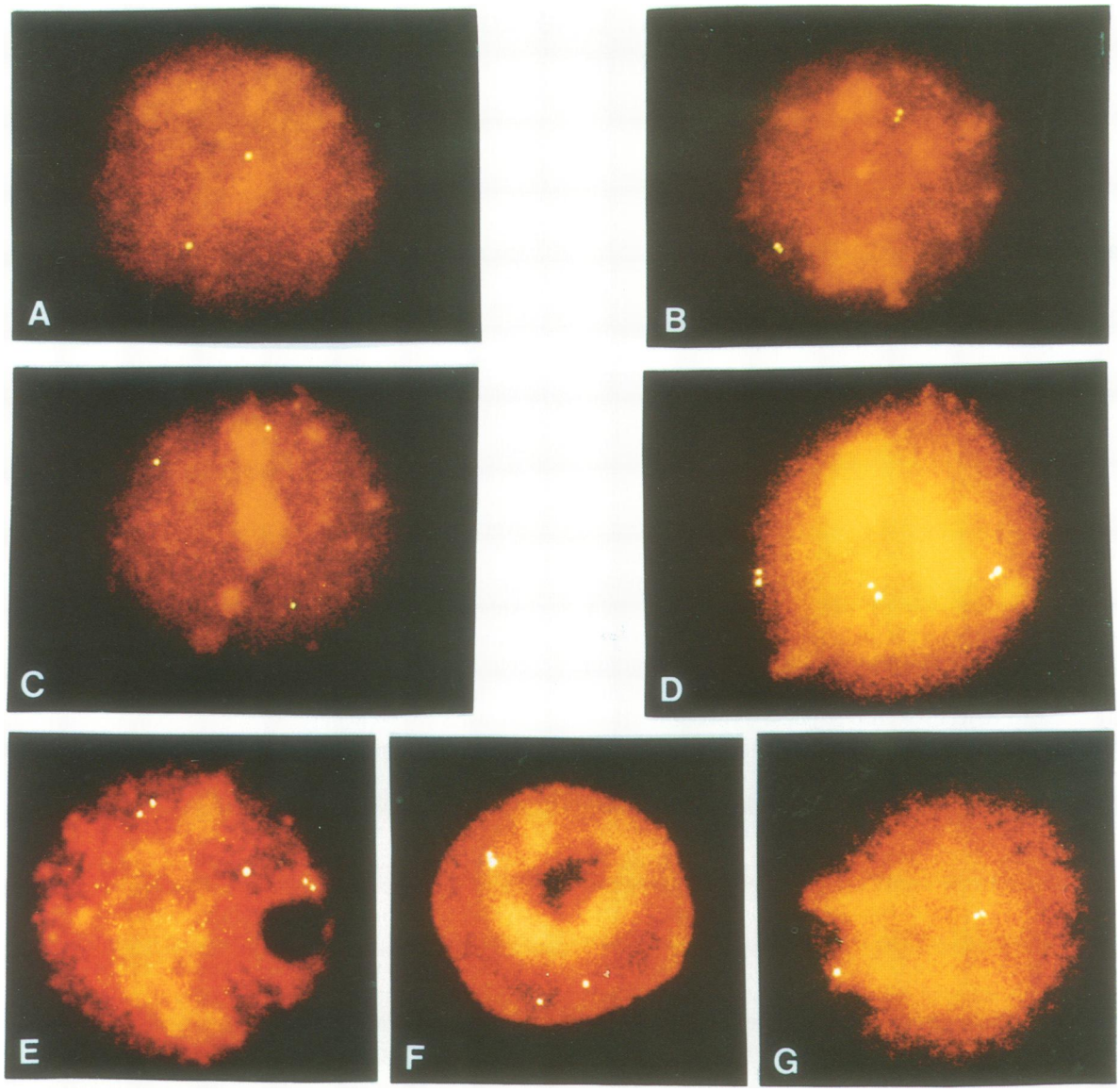


Fig. 1. *In situ* hybridization to interphase nuclei. Panels A and B show Manca nuclei hybridized with a PYGM probe. Note the presence of two types of nuclei, those with two singlet hybridization dots in which the PYGM gene has not yet replicated (**A**) and those with two doublet dots in which the gene has undergone replication (**B**). Panels C, D, E and F show the results of hybridization with the β-globin K40 probe to K-562 cells. In this line chromosome 11 has a variable number of copies. Note the presence of cells showing synchronized replication patterns (**C** and **D**) as well as two examples of non-synchronous replication (**E** and **F**). Panel **G** demonstrates hybridization of a c-*myc* probe to Manca lymphoma cells. Approximately 23% of the nuclei in this population had a non-synchronous replication pattern.

from a non-synchronous diploid cell culture is analyzed by this method, only two different patterns are observed (Figure 1A and B): either two single hybridization dots for cells in which the gene sequence has not yet replicated, or double hybridization dots for cells in which this region has already undergone replication. Using this assay, it is quite clear that genes which replicate early in the cell cycle will show a high percentage of doublets, while for a late replicating gene, most nuclei will have only singlet hybridization signals.

To test the feasibility of such an analytical method for defining replication timing, various gene probes were first hybridized to a number of different cell types (Table I). Both the CD3D gene and the muscle glycogen phosphorylase gene (PYGM) are evidently early replicating in the human lymphocyte Manca cell line, since they reveal hybridization doublets in ~60% of the nuclei. This finding is consistent with results obtained by the BrdU cell elutriation technique (unpublished results). In contrast, a representative IgH variable region probe showed a doublet count of only 30% in the same cell population, and this late replicating pattern parallels that of other variable region sequences in mouse lymphocyte lines (Calza *et al.*, 1984). All these probes yielded similar dot hybridization results in HL-60, K-562 and HeLa cells, suggesting that these particular gene loci replicate with the same temporal order in a variety of cell types. This *in situ* technology could also be used to detect cell type specific replication timing. A cosmid probe for the human β-globin locus, for example, shows 20% doublets in the non-expressing HL-60 line, while yielding 61% doublets in K-562 erythroleukemia cells, where this gene is actively transcribed.

The ability to discern the double dot pattern requires both DNA replication and sufficient spatial separation of the

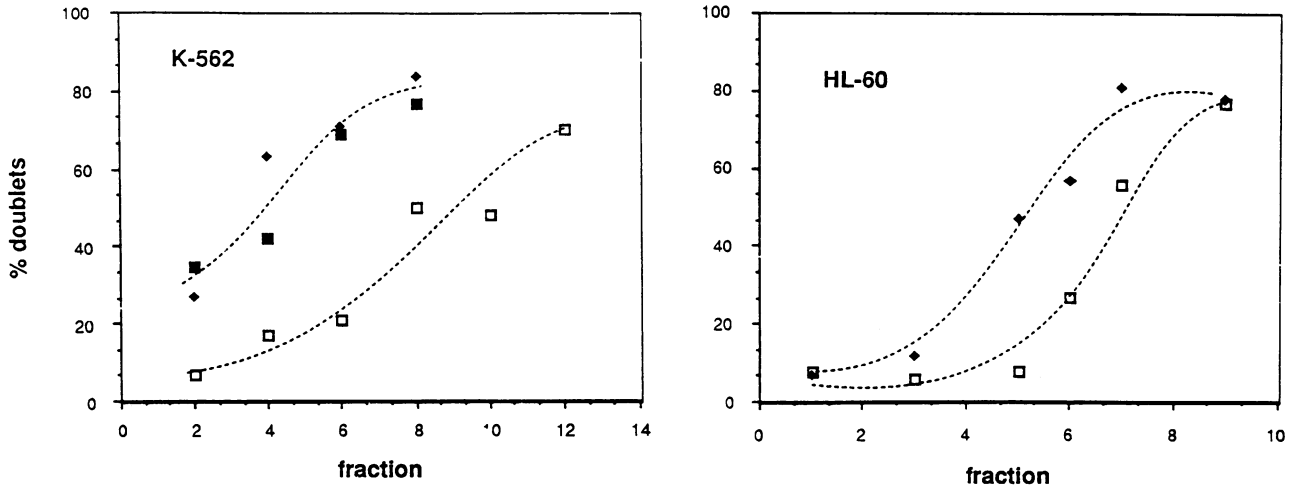
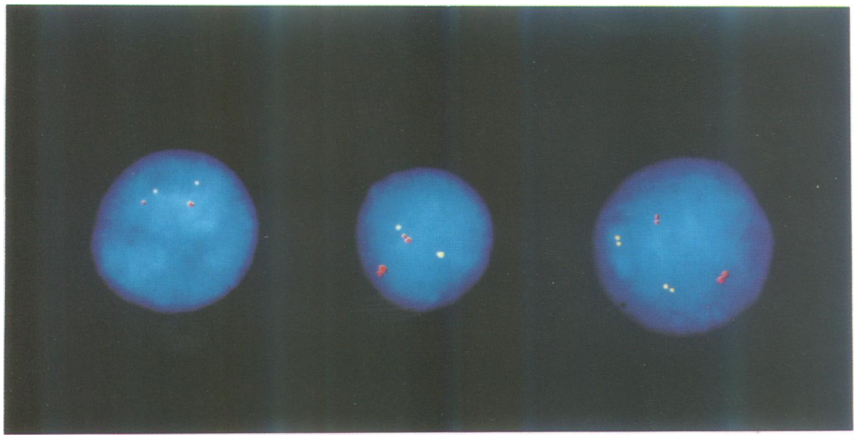


Fig. 2. *In situ* hybridization as a function of cell cycle. K-562 and HL-60 were subjected to elutriation, and nuclei from various fractions were hybridized to probes for CD3D (♦), β-globin (■) or IgH-var (□). In each case 100–200 nuclei were scored for the percentage of chromosomes showing doublets. In the case of HL-60 both homologs showed identical patterns in ~90% of the cells. In contrast, for K-562, the relevant chromosomes were present in multiple copies and the pattern of replication was asynchronous. For this reason scoring was done by counting the number of chromosomes having the doublet pattern. Thus, for these cells, the results probably represent an average of several different replication times within a limited range of S phase. While fraction 1 contains mostly G_1 cells and the last fraction mostly G_2, none of the fractions contain a pure population of cells from one defined part of the cell cycle. Thus, these fractions are merely enriched for cells in different segments of S. Futhermore, most fractions are contaminated with G_1, and this is probably the reason why even cells from the end of S do not reach a level of 100% doublets.



Replication Pattern
(percent of nuclei)

	PYGM * Globin *	PYGM * Globin * *	PYGM * * Globin *	PYGM * * Globin * *
HL-60	31	0.5	52	16.5
Manca	35	1.5	48.5	15

Fig. 3. *In situ* hybridization of nuclei using two probes. HL-60 and Manca nuclei were hybridized simultaneously with digoxigenin labeled PYGM and biotin labeled β-globin. Biotin was detected with FITC (yellow), and digoxigenin with rhodamine (red). Photographs of hybridization to Manca cells show nuclei where both genes are unreplicated (left), nuclei with replicated PYGM genes (red doublets) and unreplicated globin (yellow singlets) (middle) and nuclei with both genes replicated (only doublets) (right). For each cell type, 100–200 nuclei were scored. The percentage of doublets are recorded in the accompanying table. Both PYGM and β-globin are located on chromosome 11. Note that in both cell lines PYGM replicates prior to β-globin. In this study scoring was limited to cells showing synchrony for both individual loci.

resulting DNA chromatin strands at a particular locus. Thus, while the *in situ* data show a strong correlation between dot hybridization and replication positions in the cell cycle, further proof was required to demonstrate that this assay provides a direct measurement of replication. To this end, we monitored the conversion of specific gene sequences from the singlet to the doublet pattern as a function of cell progression through S phase. HL-60 cells were grown in culture, separated into different fractions of the cell cycle by elutriation and then assayed by the replication dot technique. With the late replicating IgH variable gene, cells early in the S phase exhibited a high percentage of singlet dots, and the number of nuclei with doublet signals increased in elutriation fractions which were enriched for cells in late S phase (Figure 2). On the other hand, the conversion from two to four dots occurred much sooner for the CD3D gene, which is known to replicate early in S phase. A similar pattern of replication kinetics was also observed in K-562 cells, where both CD3D and β-globin replicate early. These data thus demonstrate a direct relationship between *in situ* hybridization and replication timing.

Synchrony of DNA replication on chromosome homologs

From the hybridization pictures obtained in HL-60 and Manca, two cell lines which have a largely diploid chromosomal constitution, it can be seen that replication timing is highly synchronous. Thus, in ~90% of the cells examined, hybridization patterns were equivalent on both homologs. In the remaining cells, however, one can observe a hybridization pattern consisting of a doublet on one chromosome and a singlet on the other. However, this is probably not due to asynchrony in replication timing, and most likely reflects suboptimal hybridization conditions. Support for this interpretation can be derived from the fact that a similar percentage of metaphase chromosomes demonstrates hybridization on only one sister chromatid (Lichter *et al.*, 1990). An example of asynchronous replication, however, can be seen in the case of the c-*myc* gene in Manca cells, where replication can be observed on one chromosomal homolog but not the other in 23% of the nuclei (Figure 1G). This hemizygous pattern of replication is probably due to the rearrangement of the c-*myc* gene in these lymphoma cells (Hayday *et al.*, 1984). Previous studies using elutriation techniques in murine plasmacytoma cells have indeed demonstrated that the rearranged 5′ end of the c-*myc* gene replicates later than the unaltered copy (Calza *et al.*, 1984).

The synchronous pattern of replication observed for most gene markers in HL-60 and Manca cells was not a general characteristic of all cell lines. In K-562 cells, for example, chromosome 11 is present in a variable number of copies ranging from two to five, and several probes located on this chromosome revealed asynchronous replication in >50% of S phase nuclei (Figure 1E and F). It is clear that in this cell line, replication timing, determined either by elutriation or by *in situ* hybridization, must represent an average estimate. Although the reason for this asynchrony is not clear, it is possible that replication timing control requires a balance between *cis* and *trans* acting factors and the presence of additional chromosome copies may disrupt this process. One should not conclude, however, that this is a general phenomenon, since synchrony has been observed for some trisomies (Somssich *et al.*, 1984) and for the multiple satellite DNA loci in mouse cells (Selig *et al.*, 1988).

Table I. *In situ* hybridization of gene probes in different cell types

Gene	Cell type			
	K562	HL-60	HeLa	Manca
	(% Doublets)			
Globin	61	20	33	17
CD3D	70	63	47	54
PYGM	68	68	48	65
IgH-var	25	17	25	31
c-*myc*	65	–	44	–

K-562, HL-60 and Manca nuclei were prepared and hybridized *in situ* with various biotinylated probes. For each experiment, 100–200 nuclei were screened and the percentage of chromosomes showing doublet dots was recorded (see note in Figure 2). These data were not corrected for the number of nuclei in G_1 or G_2. The HeLa preparation, for example, contains a high percentage of G_1 nuclei and thus shows a relatively low percentage of doublets compared with other cell lines. c-*myc* was not analyzed in HL-60 since it is amplified in that line (Collins and Groudine, 1982), and not in Manca, due to asynchronous replication. This gene has been shown to be early replicating in K-562 cells (Iqbal *et al.*, 1987).

Ordered replication pattern of two gene loci

Analysis of replication banding in animal cells has shown that large tracts of DNA undergo replication according to a sequential time schedule. To test whether this strict programming also controls the replication of individual gene loci, HL-60 cells were hybridized simultaneously with two different probes, PYGM labeled with digoxigenin, and β-globin, labeled with biotin. Since each of these could be detected by differently colored fluorescent tags, one could observe the replication pattern of both genes in single cells. As seen in Figure 3, essentially three major types of hybridization patterns were obtained in this experiment: nuclei in which both genes had not yet replicated, nuclei in which both genes had been copied, and a large fraction of cells which revealed a replicated PYGM gene and an as yet unreplicated globin sequence. Quantitative analysis clearly shows that, in HL-60 and Manca cells, PYGM systematically replicates prior to globin in almost every cell (Figure 3). This double label approach is thus quite accurate and can be very useful for determining the replication timing of one sequence relative to another.

Replication timing zones in the cystic fibrosis gene locus

A first step in understanding the process of replication timing is to define the boundaries of the contiguous replication zones which make up the animal cell genome. The human cystic fibrosis gene domain on chromosome 7 has been well characterized; a core of 1500 kb has been restriction mapped and large portions have been cloned into plasmid, phage and cosmid constructs (Rommens *et al.*, 1988, 1989). A representative collection of plasmid clones from this central area and its flanking sequences (Rommens *et al.*, 1988, 1989; Wainwright *et al.*, 1985; Park *et al.*, 1987) provided an excellent starting point for replication mapping at the molecular level by the elutriation method (Figure 4). This initial survey outlined three large replication timing areas in K-562 cells, including a central early/middle region

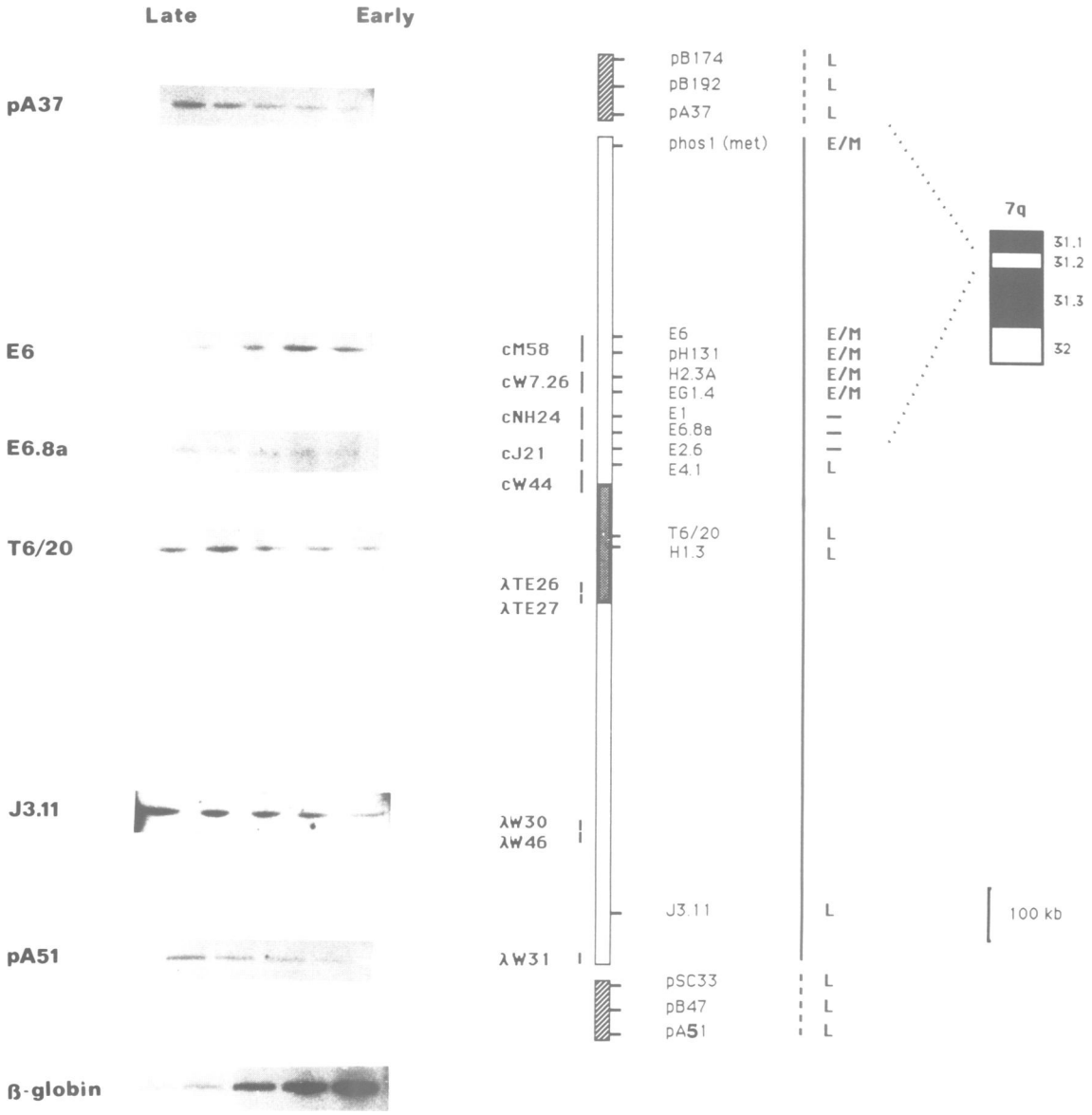


Fig. 4. Elutriation analysis of probes in the CF region. The schematic diagram shows a map of the CF region on chromosome 7 indicating the location of the gene coding sequences (shaded area). The location of plasmid probes used to assay replication time by elutriation analysis are indicated to the right of the map together with the estimated period of S when replication takes place. The entire central region shown in the diagram has been mapped by restriction analysis and much of it has been cloned (Rommens *et al.*, 1989). This is not true for the flanking regions (hatched), however, and the distance between probes in these areas is not known (Rommens *et al.*, 1988).Cosmid and phage clones mentioned in the text are also indicated to the left of the map. Replication time is shown as approximately early/middle (E/M) or late (L) but three different probes in the region from EG1.4 to E4.1 do not reveal a distinct time of replication and appear to undergo DNA synthesis throughout S. This type of behavior was also observed for other scattered probes such as pTM183, located to the 3′ side of J3.11 and pjB5-21, which is 5′ to pB192. A diagram of the G-banding pattern of chromosome 7 region 7q31-32 is shown near the physical map and all of the probes used in this study are derived from this portion of the genome (Rommens *et al.*, 1988). We estimate that the light G-band 7q31.2 may be colinear with the early/middle replicating zone at the 5′ end of the CF gene. Representative samples of blot hybridization to elutriated BruU−DNA from K-562 using specific probes are shown to the left of the map. Elutriated fractions are arranged from early S (right) to late S (left) and all of the presented results are from two separate experiments In one, we divided S into four fractions while in the other S phase cells were separated into five different fractions. It should be noted that the determination of replication time in these cells is not accurate and regions marked late could be made up of separate subzones which undergo DNA synthesis at slightly different times.

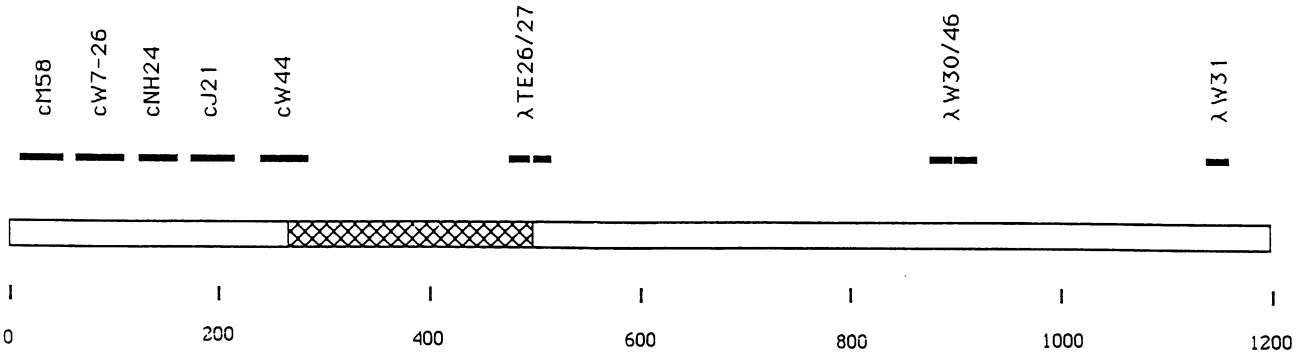
flanked on both sides by large late replicating domains, one of which contains the CF gene itself. The CF gene on chromosome 7 has been localized to band q31 (Van-der-Hout *et al.*, 1988) and all of the markers shown in Figure 4 are located within the region q31-32 (Rommens *et al.*, 1988). Thus, in a general way, these timing zones may correspond to the banding pattern of this region which contains a small light G-band (31.2) flanked by two broader dark G bands (31.1 and 31.3). This would be consistent with the observation that light G-bands co-map with early replicating DNA (Latt, 1975; reviewed by Holmquist, 1987).

This preliminary molecular replication map indicated the presence of a replication timing border 5′ of the CF gene, but elutriation analysis of numerous probes within this 100 kb region showed hybridization to all fractions of S and failed to delineate the exact boundary. In order to further

Table II. *In situ* hybridization analysis of the CF gene domain

	Replication pattern (% doublets)									
	cM58	cW7-26	cNH24	cJ21	cW44	λTE26/27	λW30/46	λW31	globin	PYGM
K-562	47	20	27	26	21	18	n.d.	19	61	68
Manca	46	16	14	13	13	13	n.d.	9	4	77
Caco-2	46	44	47	66	62	67	25	31	15	64

K-562, Manca and Caco-2 nuclei were hybridized with cosmid and phage probes from the CF region. The percentage of chromosomes having doublet dots was determined by scoring 100–200 nuclei for each preparation. In some cases adjacent phage probes were hybridized together. Each preparation was subjected to FACS analysis and the resulting cell cycle profile was used to estimate the percentage of cells in G_1 and G_2. The number of nuclei in G_1 was then subtracted from the singlet population and the number of nuclei in G_2 from the doublet count and the results presented in the table thus reflect the percentage of cells in S which show the doublet pattern. In K-562, for example, G_1 was 35% and G_2 15% of the total cell population. Following this correction procedure, the percent of doublets should directly indicate the time of replication in S. For example, a probe which shows 25% doublets, replicates at a point ~75% through S, while a probe showing 75% doublets replicates during the first quarter of the S phase. In order to obtain a more precise reading, one must also adjust the data to take into consideration the non-linear distribution of cells within the cell cycle as measured by FACS analysis. The physical locations of each hybridization probe in genomic DNA is indicated on the accompanying map.



evaluate this region we took advantage of a series of large cosmid clones to test replication timing by *in situ* hybridization (Table II). As expected from the elutriation data, DNA in the region of the gene (cW44) shows an S phase doublet content of only 21%, which is consistent with a late replication pattern, while the far 5′ cosmid (cM58) yielded 47% doublets and thus replicates at a point in the early/middle portion of S. This dramatic change in replication timing was also confirmed by a double label experiment using both cM58 and cW44 as hybridization probes on the same cells (data not shown). Further *in situ* analysis showed that the border between the two adjacent time zones maps near cosmid clone cW7-26 at a point ~100 kb upstream from the start of the CF gene. Similar results were also obtained with Manca cells.

As shown in Table II, the CF gene locus was late replicating both in K-562 and Manca, cell types which do not express this gene. Since it is thought that active genes are always early replicating, we assumed that this region must have undergone a switch in replication timing in an expressing cell type. That this is indeed the case was shown by carrying out the same set of experiments in cultured Caco-2 cells. This is an epithelial type cell line, originally derived from a colon adenocarcinoma, that expresses the CF gene (L.Tsui, personal communication). Initial hybridization analysis indicated that there are four copies of the CF locus in these cells. Probes within the coding sequence have an S phase doublet content of 60–65%. This frequency is similar to that obtained for the PYGM gene, and supports the interpretation that the CF gene itself is early replicating. In contrast, the late replicating β-globin gene showed only 15% doublets in the same population. Since both ends of the CF gene replicate at the same time in S, it is reasonable to assume that the entire CF sequence undergoes DNA synthesis in the same time interval. By using additional probes both 5′ and 3′ to the CF gene, it was possible to map the boundaries of this early replicating DNA zone. At the 5′end, the border appears to be located in the region of cosmid cNH24 not far from the switch region detected in K-562 and Manca cells. The 3′ border of this early replication time zone is situated between the end of the CF gene sequence (λTE26/27) and λ phage clones w30/46, since these latter probes, as well as the w31 region, showed a late replication pattern. Since additional cloned DNA in this region was not available, we were unable to define this border further. We thus conclude that this zone is ~400–700 kb in length and is developmentally regulated. This CF domain undergoes DNA synthesis in early S phase while at this stage the flanking regions are still unreplicated, and this creates a temporary 'replication time zone bubble' which can be visualized by *in situ* hybridization using three separate gene probes (see cover photograph).

Discussion

We have described a new approach for determining replication timing in animal cells which is both extremely rapid and simple. Unlike the use of elutriation, no pre-labeling is required and excellent results can be obtained from a relatively small number of cells. In fact, from several tissue culture plates, hundreds of slides can be prepared and stored for later use with a variety of different hybridization probes. The hybridization dot assay analysis can be performed on a number of different cell lines, including primary cultures, monolayer cells, activated human lymphocytes and slowly growing cell types. This represents an improvement over

Fig. 5. Model of DNA replication timing in CF domain. A simplified view of replication time zones in the region of the CF gene is shown in **A**. In non-expressing cells, the gene (central region) is late replicating and its 5′ end borders on an adjacent early/middle time zone. In the expressing Caco-2 cell type, however, a large module has switched to early replication, thus creating a new time zone. The progression of CF replication during S phase for two cell types is shown in **B**. In Manca or K-562 cells, the entire region shows no replication activity in early S, and the 5′ end of the domain only replicates towards the middle of S. In late S, the entire region has been copied. In Caco-2 cells the gene is located in a central domain which forms a large replication bubble in early S. During middle S this merges with the adjacent early/middle replicating zone at the 5′ end of the domain and the process of replication is only completed in late S when the 3′ flanking region undergoes DNA synthesis. The dotted lines indicate cosmid clones which are located in replicated DNA near a time zone fork. In these regions the sister chromatid strands may be in close proximity and not allow resolution by the *in situ* hybridization assay.

the elutriation technique which works most efficiently on fast growing suspension cultures. Indeed, analysis of the CF gene region in Caco-2 cells could not have been performed using other methods since these cells grow very slowly and are not amenable to elutriation.

In order to estimate the time of replication for gene sequences in S phase, corrections must be made for the number of cells in G_1 and G_2. In many cases it is possible to determine these percentages by FACS analysis or by standard cytogenetic techniques. Another possibility is to label cells briefly with BrdU to identify those cells in S phase and simultaneously assay them by *in situ* hybridization using double label fluorescent antibodies. It should be noted, however, that this correction is not always required, and it is usually possible to compare the replication timing of two different genes even without this information. Indeed, by assaying a large number of different gene loci, including some known to replicate at the beginning and end of the cell cycle, one can indirectly get an estimate for the number of cells in S phase. Further accuracy in measuring replication time can be obtained by hybridizing two separate DNA sequences within the same nucleus. In this case, the number of cells showing the replication of one without the other gives a good indication of the temporal difference between the two loci. This latter approach may be particularly useful in cases where the number of cells is limiting and there is no way of determining the size of the G_1 and G_2 pools.

While replication bands on metaphase chromosomes have been analyzed extensively, little is known about their topography or their structure−function relationship. These bands, which are delineated by BrdU incorporation, appear to be related to the chromosome architecture since there is a general correlation between early replication bands and light G bands (Latt, 1975; reviewed by Holmquist, 1987; Manuelidis, 1990), suggesting that both the structural domains and the timing domains have approximately the same topographical boundaries. The early replicating light G-bands appear to be the sites of most gene activity in the cell, since a large number of both housekeeping and tissue specific genes map to these regions (Holmquist, 1987; Bickmore and Summer, 1989). In addition, *in situ* nick translation, a technique which detects active chromatin regions, reveals a banding pattern which co-maps active domains to the same light G-bands (Kerem *et al.*, 1984).

Although each chromosome band includes several megabases of DNA and may be made up of 10–20 individual replicons, analysis of both G bands (Yunis, 1981) and replication bands (Drouin *et al.*, 1990) on less condensed prometaphase chromosomes delineates over 2000 genomic subzones. Thus, the entire genome is probably made up of a series of adjacent basic independently regulated chromosomal units which are ~1 Mb in length and are characterized by their own distinct structure and replication time. The data we have obtained on the CF gene domain suggests that this gene is located within a large region of late replicating DNA which is adjacent to an early/middle replication zone. The replication timing of the CF gene is developmentally controlled and is thus early replicating in at least one expressing cell type, Caco-2. In this line, the zone which switches its timing pattern extends over ~500 kb and is thus of the same order of magnitude as the sub-bands seen on prometaphase chromosomes. Considering the fact that animal cell replicons range in size from 50 to 300 kb (Edenberg and Huberman, 1975) the CF time zone could be made up of several individual replicons which are synchronously activated during S. Time zones of this nature probably represent basic units of regional chromosome structure having defined boundaries and *cis* acting regulatory elements which take part in the control of gene expression during development (Forrester *et al.*, 1990). In the case of the CF domain, this unit clearly has its own inherent control mechanism, since even in the expressing cell type it still replicates independently of the adjacent 5′ early/middle region.

While these data show a replication zone boundary 5′ to the CF gene in all cell types, we were unable to pinpoint the actual border elements by elutriation. Analysis of more than 20 different plasmid probes shows that the switch region lies approximately between markers EG1.4 and E4.1 since the former shows an early/middle pattern, while the latter replicates late in the cycle. Probes within this 100 kb region demonstrated a smeared pattern of equal hybridization in all fractions of S.

In situ hybridization revealed a more distinct border between time zones, but the breakpoint in K-562 and Manca cells was different from that detected in Caco-2. This discrepancy is probably due to decreased resolution of hybridization dots at points near replication time boundaries. If we assume that the switch locus in both expressing and non-expressing cells is located in the vicinity of cosmid clone cNH24, it is possible to build a model which is consistent with all of the data. In K-562 cells, DNA in the region of probe cW7-26 is early/middle replicating as indicated by the elutriation results, but appears late replicating in the interphase dot assay, probably because it is located very near the border of the adjacent late replicating time zone. In early/middle S, the newly replicated DNA strands in this region may be constrained by this boundary structure (Figure 5) and thus do not separate sufficiently to allow resolution of the hybridization dots. In contrast, probe cM58, is situated much further from the replication zone boundary, and thus shows topographically distinguishable hybridization dots in middle S. In Caco-2 cells, the situation is somewhat different due to the change in the timing order of the various replication zones. In this case, probe cW7-26 is located on the late side of the timing fork and is thus detected accurately as early/middle replicating DNA. On the other hand, since cNH24 is positioned on the replicating side of the fork its segregation in early S is not resolvable and thus appears as if it undergoes synthesis in middle S. In this cell line cosmid clone cJ21 is the first probe present at a sufficient distance from the time zone border to allow the detection of replication in early S cells.

The dot hybridization assay provides an interesting opportunity to visualize certain structural aspects of the nucleus during interphase. Very early in the cell cycle, the entire genome is present in an unreplicated form. As soon as DNA synthesis initiates, however, large 'replication zone bubbles' appear on the DNA. Depending on the replication timing of flanking regions, these bubbles may remain present in the nucleus for long time periods until they eventually merge into larger replicated structures. Although we have not done careful micrometry, it is clear that the identical gene sequences on parallel newly synthesized DNA molecules can be far apart, since they are easily resolvable by ordinary light microscopy. Thus, during the first half of S, all active sequences are probably organized in this unique bubble structure and are thus structurally, and perhaps functionally, distinguishable from the bulk of the late replicating DNA.

Materials and methods

Cells and growth conditions

Human erythroleukemia K-562 (Lozzio and Lozzio, 1975) and HeLa cells were grown in DMEM supplemented with 10% FCS. The HL-60 human promyelocytic leukemia (Collins *et al.*, 1977) and Manca human lymphoma (Hayday *et al.*, 1984) cell lines were grown in RPMI with 10% FCS. The human epithelial-like Caco-2 cell line, originally derived from a colon tumor (Fogh *et al.*, 1977) was grown in Eagle's MEM with 15% FCS. All cell lines were further supplemented with 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin and grown at 37°C in 5% CO_2. Cells used for *in situ* hybridization analysis underwent FACS analysis (Vindelov *et al.*, 1983) following harvesting in order to estimate the number of cells in S phase.

Elutriation and BrdU-DNA isolation

Elutriation was carried out with the JE-6B rotor (Beckman) using a modified version of a previously described procedure (Braunstein *et al.*, 1982). Prior to separation, exponentially growing cells ($2-6 \times 10^8$) were labeled for 2 h with 10^{-5} M BrdU, harvested by centrifugation and resuspended in 30 ml of HANKS buffer. Elutriation was performed at a fixed rotor speed of 2000 r.p.m. and cells were eluted in 100 ml fractions by gradually increasing the buffer flow rate using a peristaltic pump. In the case of HL-60 cells the flow rate gradient varied from 19.4 ml/min to 45.8 ml/min and for K-562 elution was begun at 15 ml/min and the last fractions removed at a rate of 64 ml/min. FACS analysis was used to determine the position of each eluted fraction in the cell cycle and accordingly fractions were pooled to yield four to five groups, each containing BrdU-DNA which replicated at a different interval of S phase. DNA was extracted from each sample, treated with RNase, digested with *Eco*RI and then BrdU–DNA was separated by ultracentrifugation in Cs_2SO_4 (1.42 g/ml) at 30 000 r.p.m. at 20°C for 24 h using a VTi65 rotor. Prior to centrifugation radiolabeled heavy and light density marker DNA fragments (30 000 c.p.m.) were added to each tube. Gradients were collected using a peristaltic pump and fractions containing BrdU–DNA were identified on the basis of the radiolabeled density markers. Pooled fractions were then dialyzed and DNA was precipitated with ethanol and dissolved in water at a concentration of 0.5 μg/μl. Equal amounts of BrdU–DNA (5 μg), representing different fractions of S, were electrophoresed on 1% agarose gels and transferred to a Hybond membrane (Amersham) as described (Southern *et al.*, 1975). Filters were prehybridized at 65°C for 5 min in presence of 7% SDS, 10% polyethylene glycol (mol. wt 8000) and 1.5 × SSPE (20 × SSPE is 3.6 M NaCl, 0.2 M NaH_2PO_4, 0.02 M Na_2 EDTA, pH 7.0) and hybridized for 15 h under the same conditions following the addition of denatured labeled probe (10^9 c.p.m./μg) and 250 μg/ml salmon sperm or human placental DNA.

DNA probes

The DNA probes used in this study were kindly provided by the following individuals, Lap-Chee Tsui: cosmids cM58-3.6/3.7, cW7-26, cNH24, cJ21 and cW44, phages TE26II and TE27 and plasmids pB174, pB192, pA37,

pTM183, pjB5-21, pSC33, pB47, pA51, E6, pH131, H2.3A, EG1.4, E6.8α, E2.6, E4.1, T6/20 and TE24IVH1.3 (Rommens *et al.*, 1988, 1989); Michael Dean: phages, λw30-A1, λw30-A6 and λw46-2 (Dean *et al.*, 1990); Michael Iannuzzi; phage, λW31 (Iannuzzi *et al.*, 1989); Bob Williamson: plasmid, J3.11 (Wainwright *et al.*, 1985); George Vande-Woude: plasmid phos1 from the *met* locus (Park *et al.*, 1987); Sherman Weissman: cosmid K-40 from the β-globin region (Collins and Weissman, 1984); Fredrick Alt: phage 7-1-558 from the human IgH locus (Berman *et al.*, 1988); Glen Evans: cosmid PYGM (Lichter *et al.*, 1990) and cosmid CD3D (Evans and Lewis, 1989); Michal Baniash: plasmid CD3-γ chain (Krissanesen *et al.*, 1986); and Phil Leder: c-*myc* phage (Battey *et al.*, 1983).

Fluorescence in situ hybridization

Fluorescence *in situ* hybridization was performed as described previously (Lichter *et al.*, 1988, 1990). Briefly, unsynchronized cell cultures were harvested and treated with hypotonic KCl solution (0.075 M) for 10–20 min at 37°C and fixed with cold methanol–glacial acetic acid (3:1). The cells were washed at least three times with fixative, dropped on cold slides and air dried. Denaturation was carried out by incubation in 70% deionized formamide, 2 × SSC at 68°C for 2 min, and then slides were dehydrated by a series of ice cold ethanol washes (70%, 90% and 100% for 5 min each).

RNA free cosmid or phage DNA was labeled by nick-translation, substituting dTTP with bio-11-dUTP (Sigma), or with dTTP and digoxigenin-11-dUTP (Boehringer Mannheim) in a ratio of 2:1. The critical size range of probe molecules (smaller than 500 bp and preferably 150–250 bp) was achieved by empirically varying the amount of DNaseI in the nick translation reaction. Unincorporated nucleotides were separated from the probe DNA by centrifugation through 1 ml Sephadex G-50 columns in the presence of 0.1% SDS.

DNA (20–50 ng) was mixed with human placental DNA (2–3 μg) and sufficient salmon sperm DNA to obtain a total of 10 μg of DNA in a 10 μl hybridization cocktail. After denaturation of the probe mixture (75°C for 5 min), preannealing of repetitive DNA sequences was carried out for 10 min in 37°C before application to denatured nuclei specimens. Following incubation overnight and subsequent post-hybridization washes, the specimens were treated with blocking solution (3% BSA, 4 × SSC) for 30–60 min at 37°C. All detection reagents were incubated with the specimen for 30 min at 37°C in 1% BSA, 4 × SSC and 0.1% Tween 20 and slides were then washed at 42°C three times for 5 min each in 4 × SSC and 0.1% Tween 20. Biotin labeled probes were detected with FITC-conjugated avidin DCS (1:400 dilution) (Vector Laboratories) and digoxigenin labeled probes were detected with an anti-digoxigenin antibody conjugated to rhodamine (Boehringer Mannheim) (1:100 dilution). In one case (cover photograph) we assayed three different probes. Digoxigenin was detected by rhodamine, biotin by streptavidin-infrared and DNP by FITC (Ried *et al.*, in press). Counterstaining was done using propidium iodide (200 ng/ml) in antifade solution, or with diamidinophenylidole (DAPI) for double hybridization experiments employing biotin and digoxigenin probes. Visualization was accomplished by conventional fluorescence microscopy and digital imaging was carried out as previously described (Boyle *et al.*, 1990). Photographs were taken directly from the video screen.

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